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► To cite this version:

D. A. Bronk, J. H. See, P. Bradley, L. Killberg. DON as a source of bioavailable nitrogen for phytoplankton. Biogeosciences Discussions, 2006, 3 (4), pp.1247-1277. hal-00297839

HAL Id: hal-00297839

<https://hal.science/hal-00297839>

Submitted on 7 Aug 2006

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DON as a source of bioavailable nitrogen for phytoplankton

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Received: 26 June 2006 – Accepted: 14 July 2006 – Published: 7 August 2006

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Abstract

Relative to inorganic nitrogen, concentrations of dissolved organic nitrogen (DON) are often high, even in regions believed to be nitrogen-limited. The persistence of these high concentrations led to the view that the DON pool was largely refractory and therefore unimportant to plankton nutrition. What DON is utilized was believed to fuel bacterial production. More recent work, however, indicates that rates into and out of the DON pool can be large, and that the constancy in concentration is a function of tightly coupled production and consumption processes. Evidence is also accumulating that indicates phytoplankton, including a number of harmful species, may obtain a substantial part of their nitrogen nutrition from organic compounds. Ongoing research is investigating ways to discriminate between autotrophic and heterotrophic utilization, as well as a number of mechanisms such as cell surface enzymes and photochemical decomposition, that could facilitate phytoplankton use of DON components.

1 Introduction

The largest pool of fixed nitrogen (N) in most aquatic systems is dissolved organic N (DON, Bronk, 2002). This is true even in oligotrophic environments where primary production is limited by available N. The persistence of DON in areas believed to be N-limited led to the traditional dogma that DON is largely refractory and therefore unimportant to phytoplankton N nutrition. The second part of this DON dogma is that DON is used to fuel bacterial, rather than phytoplankton, production and does so with relatively long turnover times. The objective of this paper is to highlight recent work on DON composition and bioavailability that illustrates the traditional dogmas should be discarded. Research has shown that even highly refractory compounds can be a source of bioavailable N to plankton as well as a vehicle to transport N through the coastal ocean ecosystem. Recent findings and new approaches also indicate that DON fuels a significant amount of autotrophic production.

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2 Is DON largely refractory?

2.1 The black box of DON and the elusive doughnut

Concentrations of DON are generally highest within the euphotic zone and then decrease to a low and constant value deeper in the water column (e.g. Bronk and Ward, 2005). The DON pool is generally treated like a “black box”, the composition of which is unknown but is expected to change over small space and time scales. The largest fraction within the DON box likely includes the truly refractory components that persist for months to hundreds of years (reviewed in Bronk, 2002). This fraction contains compounds such as humic and fulvic acids, porphorins, and amides and is responsible for DON's reputation as a poor source of N (Table 1). Using terminology from the dissolved organic carbon (DOC) literature, a second fraction of the pool can be described as semi-labile (Carlson and Ducklow, 1995). This fraction likely includes compounds such as proteins, dissolved combined amino acids (DCAA), and amino polysaccharides, which turnover on annual time scales. Mixed in with the refractory background, however, are the “doughnuts” of the DON world – highly labile moieties including urea, dissolved free amino acids (DFAA), and nucleic acids (reviewed in Bronk, 2002). These doughnuts turnover on timescales of minutes for amino acids (e.g. Fuhrman, 1987) to days for urea (e.g. Bronk et al., 1998) and DNA (e.g. Jørgensen et al., 1993).

The bulk of research on DON availability has focused on the labile doughnut fraction. Recent work, however, has also shown that even humic substances, considered to be highly refractory, can be a source of N. Humic substances, one of the least understood classes of aquatic DOM, make up 40 to 80% of DON in seawater (e.g. Alberts and Takács, 1999). They are operationally defined as dissolved organic material that adheres to a macroporous resin (i.e. XAD-8 or DAX-8; Peuravuori et al., 2002) at a pH of 2 (Aiken, 1988). Humic substances can be further categorized into 1) fulvic acids, which tend to be smaller (500–2000 Daltons) and are soluble in water at any pHs, 2) humic acids, which are larger (2000–5000 Daltons or larger) and precipitate from solution at pHs lower than 2 (Thurman et al., 1982), and 3) humins, which are insoluble at

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any pH.

Natural humic substances, isolated with XAD extraction, have been shown to contain 0.5 to 6% N (Rashid, 1985; Thurman, 1985; Hedges and Hare, 1987). Amino acids, amino sugars, ammonium (NH_4^+), and nucleic acid bases comprise 46 to 53% of the N associated with humic acids and 45 to 59% of fulvic acids (Schnitzer, 1985) with the remaining approximately 50% of humic-N unidentified (Carlsson and Granéli, 1993). Previous work indicates that the C to N (C:N) ratio of aquatic humic substances, isolated with XAD resin, ranges from 18 to 30:1 for humic acids and 45 to 55:1 for fulvic acids, but can vary considerably (Thurman, 1985; See, 2003). The C:N of humic substances isolated with macroporous resins, however, may not reflect the C:N ratios of humic substances in situ. During the isolation procedure humic substances are acidified to a pH of 2, thus bombarding the solution with free protons. These free protons can bump off loosely associated amino groups such that humics isolated using resins have a C:N ratio higher than humics isolated by slightly elevating the pH of the solution (thereby converting soluble NH_4^+ to volatile NH_3) and then blowing off the volatile NH_3 in a SpeedVac concentrator (See and Bronk, 2005). The presence of these dissociable amino groups is likely the bioavailable fraction of humic-N we discuss below.

2.2 Results from bioassays

The unknown composition of the DON pool makes determining its bioavailability difficult. Isotopic tracers are available for only a small fraction of the pool. As a result, bioassay approaches have been used to monitor the decrease in DON concentrations over time. One problem with the bioassay approach is it requires the ability to measure relatively small concentration changes in a large pool. Bioassays also only measure net flux within a pool, such that even large DON uptake rates could be unmeasurable if rates of DON regeneration are also high. Despite these drawbacks, a number of studies have used dark bioassays in aquatic systems to measure heterotrophic bacterial utilization of DON. In general, this work suggests that 12 to 72% of the DON pool is bioavailable on the order of days to weeks (reviewed in Bronk, 2002). In the Delaware

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and Hudson Rivers, 40 to 72% of the DON was consumed during 10 to 15 day dark bioassays (Seitzinger and Sanders, 1997). In two streams in Sweden, 19 to 55% of the bulk DON is bioavailable in short-term bioassays (Stepanauskas et al., 2000); only 5 to 18% of the DON was identified as urea, DCAA or DFAA indicating that bacteria also utilize additional organic N compounds. In the Gulf of Riga, 13% of the DON is bioavailable during 7 to 8 day bioassay experiments (Jørgensen et al., 1999).

In another study water samples were collected from rivers and estuaries along a continuum of anthropogenic modification (Wiegner et al., 2006). Dark bioassays were performed with a single bacterial inoculum to compare DON and DOC lability across a range of systems that varied with the amount of forest cover. As much as 40% of the DON was consumed over a dark 6 day incubation and up to 80% of the total N utilized by the inoculum was organic in form. Simply classifying DON as refractory clearly overlooks an important source of available N in the marine environment.

2.3 The special case of humics

The refractory nature of humic substances has also recently been challenged, and accumulating evidence indicates that coastal phytoplankton may have the ability to take up humic-N, either directly or after remineralization by bacteria (Carlsson et al., 1995, 1998, 1999). More recently, the uptake of laboratory-produced ¹⁵N-labeled humic compounds by the >0.7 μm size fraction has been observed in both riverine and coastal ecosystems (Bronk et al., unpubl. data), humic substances have been implicated as a potential source of C and N to the toxic dinoflagellate *Alexandrium catenella* (Doblin et al., 2000), and growth of another toxic dinoflagellate *A. tamarense* was shown to increase when exposed to humic substances (Gagnon et al., 2005). Uptake of humic-N into phytoplankton biomass was also measured using ¹⁵N-labeled humic substances produced in the laboratory (See and Bronk, 2005). In this experiment, non-axenic cultures of 17 recently isolated estuarine and coastal phytoplankton strains took up ¹⁵N-labeled humic-N (See et al., 2006), however, high rates of humic-N uptake were not sustained over long periods of time suggesting that a finite pool of labile N is as-

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sociated with these compounds (See et al., 2006). Two of the cultures examined were also available in axenic form. No uptake of ¹⁵N-labeled humic-N was detected in the axenic cultures suggesting that in at least these two cultures, bacterial remineralization was required to make the humic-N bioavailable.

5 2.4 Why should we care?

In a review of DON in rivers, Seitzinger and Sanders (1997) estimate that 14 to 90% of the total N in a suite of rivers around the world is organic in form. This DON represents a large source of N to the coastal zone that is currently ignored in most N loading budgets. This is especially troubling when one considers that effluent from even the most
10 efficient wastewater treatment plants contain approximately 285 μM N with roughly two thirds of that being organic in form (Pehlivanoglu and Sedlak, 2006). Its exclusion from loading budgets is based on the traditional view that DON is not bioavailable and therefore will not contribute to eutrophication. The brief review of recent studies above suggests that this traditional view is incorrect. Collectively, data from bioassays sug-
15 gest that bioavailable DON can be utilized within estuaries with water residence times on the order of weeks to months. In systems where residence times are shorter, river-ine DON will pass through the estuary and be a source of bioavailable N to coastal waters. Results from studies with individual organic compounds indicate that some fractions of DON have much quicker turnover times and consequently contribute to
20 plankton nutrition even in systems with very short residence times. It is becoming increasingly evident that a significant fraction of DON is bioavailable and contributes to coastal eutrophication and, as such, should be included in N loading budgets.

3 Are bacteria the primary users of DON?

Phytoplankton DON use has traditionally been neglected in favor of the view that bac-
25 teria are the primary consumers of DON for a number of reasons. First, phytoplankton

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are known to have specific metabolic pathways for the uptake and assimilation of inorganic N substrates such as NH_4^+ and nitrate (NO_3^- , e.g. Syrett, 1988). It is unlikely that they would evolve similar specific uptake mechanisms for the suite of organic substrates that likely exist in very low concentrations at any given time. Second, early studies of plankton uptake of ^{14}C -labeled organics indicate that the substrates enter into the bacterial-size fraction; the assumption was made that the N followed the C. More recent work has shown that some phytoplankton possess cell-surface enzymes that allow them to take up the N fraction of amino acids while leaving the C backbone available for subsequent uptake by bacteria (e.g. Palenik and Morel, 1990a, b). In other words, the N does not always follow the C. The presence of these cell-surface enzymes provides a likely mechanism whereby phytoplankton can access a broad range of organic N substrates without developing a specific uptake pathway. This discovery merits a reevaluation of the phytoplankton use of DON. Here we review methods for distinguishing bacterial uptake from phytoplankton uptake, discuss the use of tracers to measure DON uptake, and briefly review some of the more recent work on DON uptake in the field, particularly in reference to harmful algal bloom species (HABs)

3.1 Methods to measure phytoplankton and bacterial nitrogen uptake rates

Marine phytoplankton and heterotrophic bacteria are known to utilize similar pools of inorganic and organic N substrates, and this use of shared resources can result in a competitive interaction that could potentially control phytoplankton community composition. To distinguish between autotrophic and heterotrophic N uptake in an experiment, it is necessary to separate the two plankton groups. Each of the various methods that have been employed has its advantages and drawbacks. The most commonly utilized technique has been size fractionation, in which filters (in the size range of 0.7 to $1.2\ \mu\text{m}$) target the size differences between bacteria and phytoplankton (e.g. Wheeler and Kirchman, 1986; Kirchman et al., 1989; Mulholland et al., 2002; Rodrigues and Williams, 2002). Size fractionation may be easier and more time-efficient than other approaches, but can lead to inaccurate estimates of N use by phytoplankton due to

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the size overlap between bacteria and phytoplankton. Studies from a range of marine ecosystems have shown that GF/F filters can retain as much as 93% (40 to 75% on average) of the bacterial community (Lee and Fuhrman, 1987; Lee et al., 1995; Gasol and Morán, 1999; Berg et al., 2001), yet ^{15}N uptake measured on GF/F filters has traditionally been attributed to phytoplankton. Other researchers have attempted to avoid this methodological problem through the use of metabolic inhibitors such as streptomycin and cycloheximide, which block the function of ribosomes in prokaryotes and eukaryotes, respectively (Wheeler and Kirchman, 1986; Middelburg and Nieuwenhuize, 2000a, b; Veuger et al., 2004). However, this method can introduce artifacts to uptake rate measurements due to the lack of specificity and incomplete inhibition of target organisms (Oremland and Capone, 1988; Lee et al., 1992). Dark nutrient bioassays have also been used to a limited extent in field, mesocosm, and culture experiments to distinguish between phytoplankton and bacterial N uptake despite the fact that many phytoplankters are known to take up N in the dark (Gobler et al., 2002; Joint et al., 2002; Glibert et al., 2004). Finally, molecular approaches have recently gained popularity as a means of qualitatively describing the N utilization capabilities of marine phytoplankton and bacteria by measuring the activity of enzymes involved in N assimilation, such as nitrate reductase, urease, and aminopeptidase (Collier et al., 1999; Allen et al., 2001; Lopez-Lozano et al., 2002; Zehr and Ward, 2002; Dyhrman and Anderson, 2003; Fan et al., 2003b; Lomas, 2004). It is important to note, however, that the molecular techniques do not provide quantitative estimates of N uptake. It is clear, therefore, that none of the approaches described above can quantitatively distinguish between autotrophic and heterotrophic N uptake.

A promising newer approach is the use of flow cytometric sorting to physically separate phytoplankton from bacteria following tracer-addition experiments. Originally introduced into marine planktology to rapidly and precisely estimate abundance of pico- and nanophytoplankton in marine food web studies (e.g. Olson et al., 1983, 1985, 1988; Yentsch et al., 1983; Chisholm et al., 1988), flow cytometry now has a wide variety of applications in aquatic microbial ecology (e.g. Wallner et al., 1997; Dubelaar and

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Jonker, 2000; Reckermann, 2000; Veldhuis and Kraay, 2000; Vives-Rego et al., 2000; Campbell, 2001; Collier, 2004), including routine analysis of the abundance of marine heterotrophic bacteria (e.g. Monger and Landry, 1993). Using the sorting capability of a flow cytometer, researchers can isolate cells of interest based on unique optical properties conveyed by the presence of pigments, such as the red autofluorescence of chlorophyll. Following traditional ^{15}N tracer incubations, chlorophyll-containing phytoplankton can be sorted and analyzed on a mass spectrometer to directly quantify phytoplankton N uptake. This approach to measuring N uptake was pioneered by Lipschultz (1995), who used flow cytometric sorting to measure uptake rates of ^{15}N -labeled NH_4^+ and NO_3^- by coastal phytoplankton, consequently removing the effects of detritus and heterotrophs on uptake rate measurements. Similarly, Zubkov and Tarran (2005) used ^{35}S -labeled methionine and cell sorting to estimate DFAA uptake in *Prochlorococcus* and *Synechococcus* sampled from oligotrophic surface waters of the South Atlantic. Others have employed flow cytometric sorting in a similar manner to measure the primary production of specific ultraphytoplankton groups (Li, 1994) and to examine the growth rates and ^{13}C isotopic signatures of freshwater cyanobacteria and other algal groups (Pel et al., 2004). One important consideration to using flow cytometric sorting to measure plankton activity is the effect of the sorting method on the health and integrity of individual cells. To this end, Rivkin et al. (1986) examined the incorporation of radioactively-labeled compounds into phytoplankton cells both before and after sorting, and found that sorting did not affect cellular radioactivity when performed after incubations, but did in fact lead to diminished primary production estimates when cells were sorted prior to incubation. Thus, flow cytometric sorting of autotrophic cells represents a feasible approach to measure rates of phytoplankton specific N uptake.

3.2 Tracers for DON uptake

Using bioassays to quantify uptake rates of individual DON compounds is prone to the same problems encountered with bioassays that monitor the uptake of the bulk DON pool. To avoid these problems, substrates labeled with ^{15}N can be used to measure

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uptake rates even in systems where uptake and regeneration are tightly coupled. Urea and amino acids are the most frequently studied DON forms, not only because of their importance to phytoplankton and bacterial N nutrition, but because they are readily available in labeled form. These two organic substrates can also be purchased dually labeled (^{15}N and ^{13}C), and numerous individual amino acids can also be obtained. Other labeled organic tracers available include whole algal hydrosylate and lyophilized algal cells (i.e. combined amino acids), purines (e.g. guanine), pyrimidines (e.g. uracil), DNA nucleotides (e.g. guanosine monophosphate, adenosine triphosphate [ATP]), acetamide, and creatine. In addition, some researchers have examined the uptake of in situ produced ^{15}N -labeled DON derived from plankton cells grown on $^{15}\text{NH}_4^+$ or $^{15}\text{NO}_3^-$ (Bronk and Glibert, 1993; Veuger et al., 2004), or labeled humic material derived from *Spartina alterniflora* that was grown with ^{15}N -labeled NH_4^+ added to the sediment (See and Bronk, 2005; See et al., 2006).

3.3 Phytoplankton versus bacterial uptake

Traditionally the inorganic forms were thought to exclusively support primary production. Research over the past three decades, however, has shown not only that bacteria balance their DON consumption with uptake of inorganic nutrients (Wheeler and Kirchman, 1986; reviewed in Kirchman, 2000), but also that phytoplankton from various marine ecosystems use DON to meet their cellular N demands (reviewed in Antia et al., 1991; Bronk, 2002; Berman and Bronk, 2003; Veuger et al., 2004). DON uptake by phytoplankton was reported as early as 1957 (Hattori, 1957), and examined further by McCarthy in the 1970s (McCarthy, 1972a, b); however, these early studies of phytoplankton DON use focused mainly on the metabolism and kinetics of urea uptake. In contrast to an early emphasis on urea uptake at the cellular to individual species level, research has more recently focused on the ecological implications at a community level (Lomas et al., 2002).

In general, phytoplankton prefer NH_4^+ over other N sources because this reduced form requires the least amount of energy to assimilate. Nonetheless, NH_4^+ is not al-

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ways the dominant N substrate utilized by marine phytoplankton; DON uptake has been shown in some studies to satisfy a large portion of the autotrophic N requirement. For example, the contribution of urea and DFAA to total measured N uptake by coastal phytoplankton assemblages varies, but has been shown to reach as high as 80% (Harrison et al., 1985; Glibert et al., 1991; Berg et al., 2001; Veuger et al., 2004; Bradley and Bronk, unpub. data). In addition, phytoplankton communities appear to rely more heavily on reduced N, including DON, during summer months when ambient NO_3^- concentrations are typically minimal (Paerl, 1991; Berg et al., 2003a). This apparent increase in autotrophic DON use during summer could also be the result of a high bacterial demand on available dissolved inorganic N (DIN), which has been demonstrated to suppress phytoplankton biomass (Joint et al., 2002) and may even exert a selective pressure on phytoplankton species capable of exploiting available organic substrates.

Attributing the uptake of different types of dissolved N to specific phytoplankton groups in the field has been difficult at best, however, some general trends have emerged. For example, large and/or rapid inputs of NO_3^- are known to stimulate blooms of diatoms, which are physiologically suited to take advantage of and grow quickly with the onset of NO_3^- -rich conditions (Goldman, 1993; Lomas and Glibert, 1999a). Berg et al. (2003a) used linear regression analysis to link the abundance of diatoms in the Gulf of Riga (Baltic Sea) with NO_3^- uptake during spring. A negative correlation with DFAA uptake during the summer suggests that diatoms are not among the phytoplankton that exploit DON sources. Dinoflagellates and cyanobacteria, on the other hand, are typically associated with increased availability of reduced N, such as NH_4^+ and urea, relative to NO_3^- (Berg et al., 1997, 2003a; Lomas and Glibert, 1999b). In comparison, heterotrophic bacteria tend to rely predominantly on DFAA and NH_4^+ (Keil and Kirchman, 1991; Kroer et al., 1994; Hoch and Kirchman, 1995; Middelburg and Nieuwenhuize, 2000a, b), although other studies have shown substantial bacterial uptake of NO_3^- in various marine ecosystems (Kirchman et al., 1991; Kirchman and Wheeler, 1998; Allen et al., 2002).

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3.4 The special case of harmful algal blooms (HABs)

With increased evidence that a portion of the DON pool is labile it has been suggested that specific DON components could exert selective pressure over phytoplankton community composition (Paerl, 1997; Seitzinger and Sanders, 1997; Berman and Chava, 1999). Phytoplankton species capable of utilizing DON may have a competitive advantage in organically enriched environments where the DIN supply is limited. In particular, it appears that some harmful algae species are capable of using DON for nutrition and may prefer it to inorganic sources (e.g. Lomas et al., 1996; Berg et al., 1997). Specifically, dinoflagellates seem to have an affinity for organic N (Berg et al., 1997). One example is the case of the brown tide alga, *Aureococcus anophagefferens*, commonly found off the coast of Long Island, NY. Natural and additional inputs of DON, particularly urea, stimulate its growth (Gobler and Sañudo-Wilhelmy, 2001). Additionally, approximately 70% of the total N utilized during an *A. anophagefferens* bloom event was identified to be organic N (Berg et al., 1997). A high affinity for urea by another dinoflagellate, *Prorocentrum minimum*, contributed significantly to a bloom in the Neuse River estuary (Fan et al., 2003a).

Bronk et al. (2004) quantified the ability of the red tide former, *Karenia brevis*, to utilize ^{15}N labeled inorganic (NH_4^+ , NO_3^-) as well as organic substrates (urea and glutamate) and showed that although all the substrates could be utilized, the uptake of organic substrates did not exhibit Michaelis-Menten kinetic curves. At low substrate concentrations, there was an increase in the specific uptake rates as substrate concentrations decreased further. A similar situation was observed in an earlier study in which urea uptake rates at the lowest concentrations were relatively high; those results were attributed to error in the determination of ambient nutrient concentrations, which were approaching the limit of detection (Eppley et al., 1977). In the *K. brevis* study, however, ambient concentrations were fairly high such that substantial analytical error is unlikely. Another possibility is that the range of atom % enrichments that produced the elevated urea and glutamate rates were lower than the atom % enrichments used

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in the inorganic uptake, which would contribute to the elevated rates at low concentrations. However, the atom % enrichment range for the organics used in the *K. brevis* study were within the range of substrate enrichments used in numerous other studies where no increase in uptake at low concentrations has been observed. Furthermore, uptake experiments have been performed where the concentration of the substrate was held constant and the atom % enrichment was varied down to 0.01 atom % with no increase in uptake (Mulholland and Capone, unpubl. data). Another theory is that the increased rates at low concentrations are the result of a bimodal uptake mechanism. More research is necessary to define the mechanisms behind the observed deviation from Michaelis-Menten kinetics.

3.5 Why should we care where the DON goes?

Although research on DIN and DON uptake by phytoplankton and bacteria has been fairly extensive, relatively little is known about how these two groups compete for limiting N resources and how this interaction affects the community composition in the marine ecosystem. Such interactions are driven primarily by the absolute and relative availability of different N substrates in the euphotic zone, but the extent to which other factors such as light and temperature, as well as biological and physical processes, affect the outcome of this interaction remains a key area for future research. Furthermore, the relevant temporal and spatial scales at which this question is addressed will have implications with respect to the significance of phytoplankton versus bacterial N use.

In estuarine and coastal ecosystems, the relative use of organic N by autotrophs and heterotrophs will potentially affect plankton community composition, energy transfer to higher trophic levels, benthic-pelagic coupling, and the stimulation of harmful algal blooms. As discussed above, specific types of N tend to favor dominance by particular phytoplankton groups, which will affect how primary production is transferred to higher trophic levels. For example, inputs of NO_3^- into a N-limited coastal system tend to promote diatom blooms, which are either consumed by grazers or sink out of

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the water column promoting benthic-pelagic coupling. On the other hand, cyanobacteria and dinoflagellates appear to prefer reduced N substrates such as NH_4^+ , urea, and DFAA; members of these phytoplankton groups (especially toxic species) are commonly considered to be nutritionally inferior to grazers than diatoms, and thus represent a diminished supply of energy to upper trophic levels. Finally, the ability of bacteria to capitalize on available DIN during N-limited conditions may intensify cycling within the microbial loop and disconnect surface production from benthic micro- and macrofauna. Clearly, the type of N entering coastal and estuarine waters can play a significant role in altering plankton community structure, but may also affect broader scale processes determining overall ecosystem health.

In oceanic systems, the fate of N into phytoplankton versus bacterial biomass has significant implications with respect to N and C cycling on both ecosystem and global scales. New production, which is defined as primary production derived from “new” or allochthonous sources of N, has been used to quantify the energy available to support higher trophic levels (Dugdale and Goering, 1967). The f -ratio, which is the ratio of new to total (new plus regenerated) production (Eppley and Peterson, 1979). For example, the f -ratio has traditionally been calculated by dividing $^{15}\text{NO}_3^-$ uptake (i.e. new production) by the summed uptake of $^{15}\text{NH}_4^+$ (i.e. regenerated production) and $^{15}\text{NO}_3^-$ (e.g. Harrison et al., 1987). However, this method does not reflect the various organic compounds potentially utilized by phytoplankton, and also must clearly distinguish autotrophic uptake from that of heterotrophic bacteria. Following the traditional calculation, the f -ratio will be overestimated when phytoplankton are using DON sources to meet their N needs. Similarly, measuring NO_3^- uptake using GF/F filters and attributing this solely to phytoplankton will lead to overestimates in the f -ratio if bacteria are also using this inorganic substrate. Use of such biased estimates of new production in models of the global C cycle, for example, could therefore overstate the potential drawdown of anthropogenic C dioxide by the oceans.

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4 Mechanisms for accessing DON

With the accumulation of evidence that indicates phytoplankton use DON, additional effort has been directed at defining the likely mechanisms used to access this large but heterogeneous pool. In specific we review data on enzymatic breakdown, pino- and phagocytosis, and photochemical decomposition.

4.1 Enzymatic breakdown

Forms of DON are made available to phytoplankton via a number of mechanisms. For smaller molecules, such as urea and some amino acids, uptake can occur by active transport driven by a sodium ion pump or through facilitated diffusion if the concentrations outside the cell are in high enough concentrations (mM) to create a concentration gradient, something unlikely in the field. For larger compounds (>1 kDa) such as proteins, polypeptides, and humic acids, direct uptake through the use transport proteins cannot occur and phytoplankton must find other mechanisms of accessing the nutrients such as proteolytic enzymes. These enzymes are able to break down large polymers into their smaller constituent molecules, which can then be taken up by the cell. Proteolytic enzymes can be found intracellularly, attached to the outside of the cell or can be released from the cell into the surrounding media; the latter two are termed extracellular enzymes. Traditionally, it has been thought that proteolytic enzyme activity was strictly a characteristic of heterotrophic bacteria. Recent studies have shown that phytoplankton can utilize these mechanisms of nutrient acquisition, with the two primary types of proteolytic enzyme activities being amino acid oxidation and peptide hydrolysis (Mulholland et al., 2003).

Amino acid oxidases are enzymes that cleave off free amino acids and primary amines to produce extraceclular H₂O₂, NH₄⁺, and α-keto acids or aldehydes; the free NH₄⁺ is then taken up by the cell. This pathway has been quantified using the fluorescently labeled amino acid, LYA-lysine (Pantoja and Lee, 1994; Mulholland et al., 1998). One study found that three different species of phytoplankton possessed cell-

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surface L-amino acid oxidases that produced NH_4^+ , which was subsequently taken up and used for growth (Palenik and Morel, 1991). Several other species of phytoplankton are also known to possess amino acid oxidases (Palenik and Morel, 1991; Mulholland et al., 2003) and this process occurs across a range of systems (Mulholland et al., 1998). One estimate is that 20% of the DON utilization in coastal and oceanic environments may be due to phytoplankton utilization of amino acid oxidases (Mulholland et al., 1998).

Peptide hydrolysis is another proteolytic process in bacteria and phytoplankton whereby the peptide bonds of proteins and polypeptides are broken, liberating smaller peptides and amino acids. A study by Mulholland et al. (2002) found high rates of peptide hydrolysis in axenic cultures of *A. anophagefferen*. Leucine aminopeptidase (LAP) is one class of proteolytic enzyme capable of hydrolyzing peptide bonds. LAP is capable of breaking down a number of substrates, but has a preference for the terminal N of non-polar amino acids such as leucine (Langheinrich, 1995). Even if phytoplankton are incapable of producing their own extracellular enzymes, they may still benefit from those released by bacteria or by liberation of bacterial intracellular enzymes. Proteolytic enzymes may be freed from bacteria by cell lysis or released due to grazing on bacteria (Chrost, 1991). There is some evidence that phytoplankton, in association with bacteria, may be able to utilize monomers derived from bacterial enzymatic activity. For example, high aminopeptidase activity (10 to 90% of activity) has been found in $0.2\ \mu\text{m}$ filtrates (Jacobsen and Rai, 1991), indicating that the enzymes were liberated from the bacteria but could still have the capability to break off free amino acids that could then be used for uptake by other organisms.

In a more general sense, the use of cell surface enzymes capable of cleaving amino groups is also a potential mechanism used to access the approximately 50% of humic-N that is in the form of amino acids, amino sugars, NH_4^+ , and nucleic acid bases (Schnitzer, 1985). It is likely that these enzymes are capable of cleaving the N from a wide range of additional DON compounds allowing phytoplankton to, in effect, “farm” N from these compounds while leaving the remainder of the compound external to the

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cell (Mulholland et al., 1998; Berg et al., 2003b; Stoecker and Gustafson, 2003).

4.2 Pinocytosis and phagocytosis

DON can also be taken up by some phytoplankton by two similar processes: pinocytosis and phagocytosis. Pinocytosis is a process where the cells ingest dissolved macromolecules from the medium outside the cell. Phagocytosis is an engulfing of particulate material from outside the cell. Cells undergoing pinocytosis expand a vesicle from the plasma membrane that engulfs the molecule and brings it inside the cell. The molecules can then be accumulated inside a vacuole. Prior studies with dinoflagellates, euglenoids, and chlorophytes have found pinocytosis to be a common method for ingesting dissolved macromolecules (Kivic and Vest, 1974; Klut et al., 1987). In one case, the HAB-forming dinoflagellate, *Alexandrium catenella* directly took up fluorescently labeled dextran (Legrand and Carlsson, 1998). Other similar markers like lectins and peroxidases have also demonstrated pinocytosis in the flagellates *Amphidinium carterae* and *Prorocentrum micans* (Klut et al., 1987). Phagocytosis by marine and freshwater phytoflagellates is well documented (e.g. Sanders and Porter, 1988) and occurs under a wide range of light and nutrient regimes. In phytoplankton, phagotrophy is also seen in the photosynthetic bloom-forming dinoflagellates *Heterocapsa triquetra* and *Ceratium furca* (Legrand et al., 1998; Smalley et al., 1999). Supplementing their nutrition in this manner could give some phytoplankton a competitive advantage over other strictly photoautotrophic organisms and would allow nutrient acquisition even during unfavorable conditions (Li et al., 2000a, b). The ability to utilize additional modes of nutrient acquisition could make one species more successful than another leading to bloom formation (Smayda, 1997; Stoecker et al., 1997). As a result pino- and phagocytosis are of particular interest for those studying HABs.

Furthermore, pinocytosis and phagocytosis are two processes that could potentially be used to access N associated with large humic compounds. Experiments have been conducted using dual-labeled humic substances (^{15}N , ^{13}C) based on the assumption that if pino- or phagocytosis was used to take up the humics, both ^{15}N and ^{13}C would

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be taken up in the same stoichiometric ratio as they appear in the labeled humics. In experiments with three coastal phytoplankton strains known to take up humic-N, *Synechococcus* sp., *Amphidinium carterae*, and *Thalassiosira* cf. *miniscula*, no uptake of ^{13}C was measured indicating that the humics were not taken up whole (See et al., 2006).

4.3 Photochemical decomposition

An alternative method for the acquisition of N from humic substances is photooxidation followed by uptake of the N photoproducts. Due to their aromatic nature, humic substances are particularly photoreactive to ultraviolet (UV) light and release inorganic N (Bushaw et al., 1996; Bushaw-Newton and Moran, 1999; Kieber, 2000) and small labile organic molecules (e.g., amino acids and urea) into the surrounding environment when exposed to UV radiation (Amador et al., 1989; Jørgensen et al., 1998; Bushaw-Newton and Moran, 1999). Of these compounds, NH_4^+ generally has the greatest observed rates of production (Bushaw et al., 1996; Gao and Zepp, 1998). Photochemical release has been observed in a number of locations including an estuary in Georgia (Bushaw et al., 1996; Gao and Zepp, 1998) and a humic-rich lake in Venezuela (Gardner et al., 1998). Photodegradation of DOM increases the availability of more labile N sources to phytoplankton and bacteria.

4.4 Possible humic shuttle

In addition to the direct utilization of DON compounds, it is possible for some high molecular weight compounds to act as a transport mechanism. Humic acids contain a high concentration of cation exchange sites, allowing them to retain and exchange numerous ions from the surrounding waters. For example, humic compounds in marine clay sediments can account for a large percentage of the cation exchange capabilities of the marine benthic environment (Rashid, 1969). Additional studies show that NH_4^+ can be adsorbed to humics in the estuarine and nearshore environment (Rosenfeld,

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1979; Gardner et al., 1991). This NH_4^+ can then be released into the surrounding water column as the cation exchange sites on the humic compound are exposed to increased salt concentrations (Rosenfeld, 1979; Gardner et al., 1991, 1998; See and Bronk, 2005).

5 In coastal systems, humic and NH_4^+ concentrations are often high in the low salinity upland river marshes. In these locations, NH_4^+ can adsorb to the cation binding sites found on the humic structure. As humic compounds are flushed downriver and interact with more saline waters the adsorbed NH_4^+ can be replaced with salt ions on the humic structure making the newly released inorganic N directly available to phytoplankton and bacteria for uptake. Studies of aquatic humics collected from three U.S. east coast
10 rivers (York, Altamaha, and Satilla) confirmed the ability of humic material to adsorb and subsequently release NH_4^+ to the environment along an estuarine salinity gradient, thus acting as a “shuttle” delivering labile N to more N-limited mid-saline regions of the estuary and coastal ocean (See, 2003; See and Bronk 2005). Preliminary results
15 indicate that this “humic shuttle” is capable of releasing approximately $1\ \mu\text{mol}$ of N per liter of water passing through the estuary over the course of the year ($\sim 77 \times 10^6$ g of N into the Altamaha estuary) and is a previously unrecognized source of N to the mid-saline estuary and coastal ocean (Beck et al., 1974; Alberts and Takács, 1999).

5 Conclusions

20 In the past DON was viewed as a large refractory pool that only contributed to bacterial production over relatively long time scales. Today the DON pool is more accurately envisioned as being composed of at least two distinct pools – a large refractory pool, which likely turns over on timescales of months to years and the “doughnut” fraction composed of highly labile compounds, such as amino acids and urea, that likely turns
25 over in minutes to days. This labile fraction has been shown to contribute to autotrophic production, in addition to the more traditionally viewed bacterial fraction. Recent findings on DON utilization argue strongly for inclusion of DON in N loading budgets to

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coastal zones and estuaries, particularly as they may contribute to growth of the primary producers.

Acknowledgements. The writing of this manuscript was supported by NSF grant OCE-02218252 to D. A. Bronk. This is contribution number 2760 of the Virginia Institute of Marine Science, The College of William and Mary.

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Table 1. Labile, semi-labile and refractory components of the DON pool.

Labile	Semi-labile	Refractory
Urea	Proteins	Humic acids
DFAA	DCAA	Fulvic acids
Nucleic acids	Amino polysaccharides	Porphorins
Methylamines	(e.g. chitins and peptidoglycans)	Amides